REMARKS

The above amendment added no new matter and is merely made to more accurately describe and claim the invention.

It is respectfully submitted that the application is now in condition for allowance, which allowance is respectfully requested.

The Commissioner is authorized to charge any fee or credit any overpayment in connection with this communication to our Deposit Account No. 11-1449.

Respectfully submitted,

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CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 on June 4, 2002.

Angel Webb

VERSION WITH MARKINGS TO SHOW CHANGES MADE

SPECIFICATION:

Page 9, Lines 26-33 and Page 10, Lines 1-7:

Figure 3 is a photograph demonstrating the specificity of rabbit antiserum to ebaf by Western blot analysis; in each lane, 10μg of extracted endometrial proteins was resolved in a 15% gel by SDS-PAGE and then subjected to Western blot analysis; the blot was probed with the antiserum alone (left lane) and with the antiserum-preincubated with a 100 molar excess if the CASDGALVPRRLQHRP-amide (Seq. ID. No. 3); the antiserum was used at 1:250 dilution of a solution containing 0.8 mg protein/ml; the peptide was used at 0.32 mg/ml; for lane legend see Table 1; several immunoreactive bands (55/60, 41, 31, 25 kD) were detected by the antiserum (left lane); the immunoreactivity of the antiserum markedly reduced by the pre-incubation with the peptide (right lane); the size of the proteins are shown in kilodaltons;

Page 15, Lines 33-34 and Page 16, Lines 1-14:

Figure 17 is a photograph showing, in the Upper panel: Western blot analysis of the endometrial proteins; lane 1: molecular weight markers. 75 μg of placental proteins (lane 2), and cytosolic proteins of late proliferative (lanes 3-4) and the late secretory (lanes 5-7) endometria were subjected to Western blot analysis using the affinity purified rabbit antiserum against a peptide (CASDGALVPRRLQHRP-amide) (Seq. ID. No. 3) at the C terminal domain of the ebaf; a 41 kD protein was revealed in the placenta as well as endometria; at least three additional bands (bands 1-3) were also observed in endometria, band 3 is about 55 kD; Lower panel: the relative optical densities of the bands identified in the endometria were measured by laser scanning densitometry; the relative optical densities of the bands (1-4) is markedly increased during secretory phase; LP: late proliferative; LS: late secretory;

Page 16, Lines 33-34 and Page 17, Lines 1-18:

Figure 19 is a photograph showing the demonstration of immunoreactivity and specificity of the rabbit anti-serum to ebaf by Western blot analysis; A: in each lane, 10 micrograms of extracted endometrial proteins was resolved in a 15% gel by SDS-PAGE and then subjected to Western blot analysis; the blot was probed with the anti-serum alone (left lane) and with the antiserum-preincubated with a 100 molar excess of the CASDGALVPRRLQHRP-amide (Seq. ID. No. 3); the antiserum was used at 1:250 dilution of a solution containing 0.8 mg protein/ml; the peptide was used at 0.32 mg/ml; several immunoreactive bands (~55/60, ~41, ~33, ~25 kD) were detected by the antiserum (left lane); immunoreactivity of the antiserum markedly reduced by its pre-incubation with the peptide (right lane); B: 250 micrograms total protein from a menstrual day 1 endometrium was immunoprecipiated using the rabbit antiserum to ebaf; the detected proteins bands were similar in size to those identified by Western blotting; in addition, an additional, ~12kD, band was also detected, the size of the proteins is shown in kilodalton;

Page 27, Lines 20-27:

One additional embodiment of the present invention is the development of an antisera for *ebaf*. An antibody with specificity is useful in determining the presence of *ebaf*, or an ebaf variant, in a sample. By variant, it is meant that a[n] variant which is functionally relevant. Further, the peptide CASDGALVPRRLQHRP-amide (Seq. ID. No. 3), as demonstrated in the examples below, has been shown to be effective in the development of such an antisera.

Page 47, Lines 11-33 and Page 48, Lines 1-29:

The polyclonal rabbit antibody raised against a synthetic peptide at the C terminal domain of the ebaf reacted with a major 41 kDa protein in the placenta as well as the endometrium. In the case of lefty, which is the mouse homologue of the human ebaf, the expression of the protein in 293T cells led to formation of a non-secretory, 42 kDa protein which is the size of the pre-pro-protein (Meno *et al*, 1996). The predicted size of the pre-pro-protein of the ebaf is 41 kDa. The members of the TGF-β super family are synthesized as pre-pro-proteins which are cleaved at RXXR (Seq. ID. No. 2) sites to release the mature form of the protein. The predicted protein of ebaf exhibits two such RXXR sites (Seq. ID. No. 2) which are located at amino acid residues of 73-76 and 131-134 respectively (Kothapalli *et al*, 1997). If one of these sequences is the cleavage site, a mature protein of 294 and 236 amino acids should be produced. The deduced amino acid sequence of lefty also contained two potential cleavage sites at amino acid residues of 74-77 and 132-135, yielding mature proteins of 291 and 233 amino acids (Meno et al, 1996). Therefore, the processing of the protein and cleavage in the first versus the second RXXR site are dependent on the cell type that expresses the protein. Expression of lefty in BALB/3T3 cells led to the release of processes 25 and 32 kDa proteins into the conditioned media of the cell cultures which corresponded to cleavage at the first and second RXXR (Seq. ID. No. 2) sites respectively (Kothapalli *et al*, 1997). Such products were not observed in the Western blot analysis of cytosolic endometrial proteins. This inability to detect secreted proteins is attributed to the rapid release of the secreted proteins from endometrial cells. TGF-β are secreted proteins and ebaf has a signal peptide suggesting that the processed protein is also [be] secreted (Kothapalli et al, 1997). Western blot analysis revealed the presence of additional bands which were larger than the 41 kDa. Such products are produced as a result of post-translation modifications. It has been shown that the ebaf protein contains at least one potential glycosylation site (Kothapalli et al, 1997). The presence of this potential glycosylation site in the ebaf suggests that the protein is susceptible to modifications after translation (Kothapalli et al, 1997). Northern blot analysis or *in situ* hybridization did not reveal the presence of detectable ebaf mRNA in the proliferative

endometria. This does not rule out, however, a low level of gene transcription and translation. This may account for the low level of ebaf protein detected by the Western blot analysis. On the other hand, the protein once produced may have a long half-life, making it detectable by the Western blot analysis. Nevertheless, consistent with the data from Northern blot analysis and *in situ* hybridization, both Western blot analysis and immunohistochemical localization of the ebaf showed the presence of significantly more ebaf protein in the late secretory as compared to the proliferative phase.

Page 54, Lines 23-33, Page 55, Lines 1-34, and Page 56, Lines 1-21:

Various [F]forms of ebaf protein In The Endometrium, Endometrial Fluid and Serum During The Menstrual Cycle: The size of the ebaf precursor protein is 41 kD. ebaf protein contains three RXXR cleavage sites which conform to the minimal requirement for efficient processing by Furin, a ubiquitous prototypical mammalian kexin/subtilisin-like endoproteinase involved in the proteolytic processing of a variety of proteins including those within the ebaf super family. If all these sites ate cleaved, then products of the molecular weights of 32.3, 25.7 and 12 kD proteins are expected to be secreted (Table 2). To detect such proteins in human endometrium, an antiserum was raised against the peptide CASDGALVPRRLQHRP-amide (Seq. ID. No. 3) at the COOH terminal of the ebaf protein. When purified on a peptide column, this antibody reacted in an ELISA with the peptide (ELISA reading: 23, 500) and its reactivity could be inhibited by pre-incubation of the antibody with 100 fold excess of the peptide (ELISA reading: 700). The antiserum reacted with a number of endometrial proteins on the Western blot analysis which included a 41 kD protein, as well as 31, 25, (25/27) and 55 (55/60) kD protein bands (Figure 3). These bands were not detected when the antibody was omitted during the immunostaining procedure. When adequately resolved, the 41 kD protein appeared as a doublet. The immunoreactivity of the antiserum with these bands was completely abolished by pre-incubation of the antiserum with 100 fold excess of the peptide (Figure 3). These findings show that the antibody reacts specifically with ebaf. However, the predicted 12 kD protein was not

detected. To show that such protein exists in human endometrium, the endometrial proteins were immuno-precipitated by the antiserum. The immunoprecipitated proteins were subjected to SDS-PAGE and examined by Western blot analysis. In addition to the bands detected by Western blot analysis, an additional 12 kD protein band was detected (Figure 4). To show the temporal pattern synthesis and/or secretion of endometrial ebaf protein throughout the menstrual cycle, Western blot analysis was carried out on a number of endometria obtained from various phases of the menstrual cycle (Figure 5). The immunoreactive ebaf bands (55/60, 41, 31, 25 kD) were detected during the menstrual cycle (Figure 5). However, this immunoreactivity was greatly reduced during the "implantation window" (Figure 5). The disappearance of the smaller in size bands was more pronounced than that observed for the larger protein band (55/60 kD), apparently due to excessive amount of this latter protein. ebaf has a signal peptide and appears to be a secreted cytokine. Therefore, the endometrial fluid and the sera of normal fertile subjects for the presence of immunoreactive ebaf was examined. The immunoreactive ebaf bands were detected both in the endometrial fluid (Figure 6) as well as in sera (Figure 7). The ebaf bands 41, 31 and 25 kD were relatively more abundant during the late secretory/menstrual phase both in the endometrial fluid (Figure 6) as well as in the serum (Figure 7). The amount of these proteins was particularly low in the serum during the early and mid-secretory phase (Figure 7). To further validate these findings, sera were obtained from normal fertile women on different days after the lutenizing hormone (LH) surge. The amount of the immunoreactive ebaf bands were markedly reduced during days 5-9 post LH surge and were elevated in the days 10-14 post LH surge. In the male sera, the 55 (55/60), 41 and 25 kD proteins were detected, however, the 31 kD form of the ebaf protein was not found in these sera (Figure 7).

Page 62, Lines 14-33 and Page 63, Lines 1-4:

PCR was carried out as described using the 5' primer (B2P9): TCAGCGAGGTGCCCGTACT (Seq. ID. No. 4) and 3' primer (B2P1):

AGTTCTTAGAGCTGAAGCC (Seq. ID. No. 5). Briefly, 1 μg of reverse transcribed RNA was amplified with 0.5-1 μM of each of the 5' and 3' primers specific for *ebaf* in a 50 μl reaction volume containing 1.25 U AmpliTaq DNA polymerase, 1.25 mM $MgCl_2$, 20 μM of each of dATP, dCTP, dGTP, dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and sterile distilled water. Negative control tubes received non-reverse transcribed RNA to verify absence of contaminating DNA. Positive control tubes received all the reagents in the reaction mixture, however, the primers used were specific for β-actin. The reaction mixture was over-layered with 50 μl of mineral oil and the tubes were heated for 5 minutes at 95°C. After initiation of temperature cycling, samples were amplifies for 25 cycles. The denaturation temperature was 95°C for one minute, annealing temperature was 55°C for one minute and the extension temperature was 72°C for 2.5 minutes. Temperature cycling was concluded with a final extension at 72°C for ten minutes and the reaction products were maintained at 4°C. Amplified products were resolved in a 2% agarose gel and the bands were visualized by ethidium bromide staining. The 123 basepair DNA ladder was used as molecular weight markers.

Page 65, Lines 22-34 and Page 66, Lines 1-30:

Premature Expression Of ebaf protein In The Endometria Of Infertile Patients: To localize the ebaf protein in endometrium, two polyclonal rabbit antisera were raised against a sequence (CASDGALVPRRLQHRP) (Seq. ID. No. 3) that resides at the carboxy terminal end of the express ebaf. The pooled polyclonal anti-sera, affinity purified over a peptide column, reacted with a 41 kD protein on the Western blot analysis of both placenta as well as endometria, which is the size of the pre-pro-protein (unpublished data). Immunohistochemical stainings were first carried out using the same antiserum on frozen sections of a late secretory endometrium known to express ebaf mRNA by both Northern blot analysis and by *in situ* hybridization. Immunoreactivity could be observed in the stroma and to a lesser extent in the endometrial glands of the upper functionalis (Figure 17A). To show that such

immunoreactivity is specific, the primary antibody was omitted. In the absence of the primary antibody no specific immunoreactivity could be observed (Figure 17B). Furthermore, the primary antibody was pre-incubated with progressively higher amounts of the synthetic peptide. Pre-incubation if the primary antibody with an appropriate concentration of the synthetic peptide (14- μg/ml) completely abolished the immunoreactivity (Figure 17C). Additional immunostaining using late secretory and menstrual endometria showed the same pattern of immunostaining. However, frozen sections of endometria dated to early and mid-secretory phase failed to reveal any positive immunoreactivity (Figure 17D). Then, the frozen sections of endometria obtained from early to mid-secretory phase of infertile patients were subjected to the immunostaining (Table 7). Three different patterns of immunoreactivity were detected. In some endometria, both the endometrial glands and stroma strongly exhibited a positive immunoreactivity (Figure 18A, Table 7). In some endometria, primarily a stromal pattern of staining was visualized (Figure 18C, Table 7). In other endometria, a glandular pattern of immunostaining emerged (Figure 18E, 18J, Table 7). Yet, in some endometria, the immunoreactivity was not easily detectable (Figure 18I). The sections that were immunostained in the absence of the primary antibody did not show any evidence of immunoreactivity (Figure 18B, 18D, 18F, 18H, and 18J).

Page 71, Lines 8-33 and Page 72, Lines 1-15:

A monoclonal and rabbit antisera were raised against the peptide CASDGALVPRRLQHRP-amide (Seq. ID. No. 3) at the COOH terminal (Tabibzadeh et al, 1998) and to acetyl-DRADMEKLVIPAC peptide (Seq. ID. No. 6) at the NH2 terminal of the *ebaf* (Figures 25-26). Rabbit antiserum to CASDGALVPR RLQHRP-amide (Seq. ID. No. 3) was purified on a peptide column. The antibody reacted in an ELISA with the peptide (ELISA reading; 23,500) and its reactivity could be inhibited by pre-incubation of the antibody with 100 fold excess of the peptide (ELISA reading; 700). The proteins derived from endometria were subjected to Western blot analysis using this (Tabibzadeh et al, 1998) and the antibody to the NH2 terminal of the *ebaf*. Both

antibodies reacted with a 41 kD protein which is the predicted size of the precursor protein in endometrium, endometrial fluid and placenta (Figure 25, Tabibzadeh et al, 1998). This immunoreactivity could be inhibited with excess amount of peptide and could not be seen with preimmune sera (Figure 25A). The C-terminal antibody reacted with ~25, and ~33 kD proteins in endometrium (Figure 25A). When the endometrial proteins were immunoprecipitated, the 12 kD protein was also detected (Figure 25B). Using this antibody, *ebaf* was detected in human endometrium throughout the menstrual cycle (Tabibzadeh et al 1998). To verify the size of the *ebaf* protein, we transfected NIH 3T3 cell line with the sense and antisense cDNAs of *ebaf* and then the cells and the conditioned media were subjected to Western blotting. The blot was probed with the affinity purified rabbit antiserum to *ebaf* and the monoclonal antibody to C terminal end of *ebaf*. As shown in Figure 26, there was no reactivity with the cell lysates and conditioned media of cells transfected with the antisense cDNA whereas the sense cDNA induced the expression of the *ebaf* precursor in the cells. The conditioned media of cells transfected with sense cDNA also contained the precursor protein as well as expected mature secreted *ebaf* proteins in the size of ~12, ~25, and ~33 kD. These findings confirm that the *ebaf* exists as a precursor protein in the size of 41 kD and secreted proteins in the size of ~12, ~25, and ~33 kD (table 1) and is present in the endometrium, endometrial fluid and serum.

Please insert Sequence Listing after Page 81, after Table 7, of the Specification.